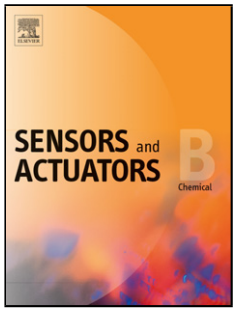

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Real time aroma reconstruction using odour primaries

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Highlights

- A procedure was developed to reconstruct natural aromas by blending odour primaries
- An aroma synthesiser was built to show the feasibility of this procedure
- The concept was illustrated by reconstructing the aroma of five *Citrus* species
- The classification accuracy of the reconstructed aromas was 73%
- The aroma synthesiser can be used for real time applications

Abstract

A methodology was developed to derive odour primaries based on the actual composition of a set of target aromas to be reconstructed. These odour primaries are mixtures of pure aroma components. The methodology was applied to reconstruct the aroma of five citrus species (lime, lemon, orange, grapefruit and mandarin) in real time using an aroma synthesiser with four nozzles dispersing odour primaries blended using 12 pure odorants that was built for this purpose. The composition of the actual and reconstructed citrus aromas was analysed using gas chromatography – mass spectrometry (GC-MS). Citrus species could be discriminated based on their headspace volatile composition. The aroma synthesiser was shown to be able to blend aromas in real time. The theoretical and actual correct classification accuracies of the reconstructed aromas were 80% and 73%, respectively. The required number of primaries and also pure odourants to construct these primaries was higher than anticipated, suggesting that further research with respect to the dimensionality of aroma spaces is required.

Keywords

Aroma synthesiser, odourant, aroma reconstruction, *Citrus*, headspace, aroma space

Introduction

Aroma synthesis is an ancient art that has been practiced by mankind since ages through cooking and the creation of perfumes and fragrances for a wide range of applications. Cooking aims, amongst others, at combining raw plant or animal based raw ingredients and transforming them through various processes into a product that has a pleasant aroma. Perfume design is a complex process that integrates natural essential oils and fragrances with artificial ones and needs input from both perfumers and chemists.

Aromas are often very complex and may consist of many odourants – volatile compounds that interact with the odour receptors in the olfactory epithelium inside the nose cavity. The coffee aroma, for example, comprises more than 800 odourants [1] with a wide range of functional groups. Of these, 29 components were identified as being responsible for most of the roast and ground coffee aroma, and only 13 of them proved to have a key contribution [2-4]. The process of creating novel aromas is complicated by the enormous number of volatile odourants but also by the fact that the human olfactory system is synthetic rather than analytical. The large number of signals transmitted by olfactory receptor neurons in the olfactory epithelium upon stimulation by hundreds of odourants are integrated to one distinct aroma impression [5]. Studies have shown that humans typically can only identify up to four single components in an odourant mixture [6,7] and that the sensitivity varies widely from non-detection to parts per billion (ppb), depending on the odourant, panelists and method of threshold determination. For example, limonene – the volatile responsible for the typical aroma of *Citrus* fruit – has an odour threshold of 4 to 3000 ppb in water, while 2-isobutyl-3-methoxypyrazin that has a green pepper-like aroma can even be detected at concentrations as low as 0.001 to 10 ppb in water [8]. In the remainder of this manuscript we will use the term ‘aroma’ to both describe the human sensation caused by a mixture of odourants emanating from a food, as well as the mixture of odorants that evokes an aroma perception.

Recently there is an increased interest in real time aroma synthesis. Such a controlled delivery of odours has a great potential in the film industry, gaming, advertisement, healthcare and even to art. The smell of mud and gunpowder during a war scene or the smell of gasoline and burned rubber during a pursuit scene would definitely enhance the realistic experience of the storyline. Also, several companies provide pleasant and distinct smells to help sell products and to strengthen brand strategies

(Ambius, Aartselaar, Belgium; ScentAir, Taplow, UK; Mood Media, Naarden, Netherlands). Aroma synthesis has also inspired art through aroma concerts on an Olfactiano (Peter De Cuypere, http://www.scentconcerts.com/) and the design of odour emitting clothing or jewellery (Jenny Tillotson, http://www.ceb.cam.ac.uk/directory/jenny-tillotson). Hundreds of patents exist on aroma evaporators and sprays, but only a few companies have addressed real time aroma synthesis, including MicroScent (Wilmington, DE); Aerome (Cologne, Germany); Aromajet (Plano, TX); Osmooze (Loriol-sur-Drome, France). They employ diverse physical mechanisms like capillarity, evaporation, nebulisation, spraying and piezo electric evaporation in their systems. The Nakamoto lab investigated odour recording with an electronic nose, followed by its reproduction by means of a mixing device using liquid basic aroma samples [9-11]. Kim et al. [12] described an array of tiny reservoirs constructed in PDMS, each filled with a different odourant. A 2D grid of electrical wires was used to heat individual reservoirs and expel the odourants through a tiny hole at the top of the reservoir. An excellent overview of aroma synthesisers, also called olfactory displays, is given in reference [13].

Although any aroma can in theory be reconstructed by blending its constituent odourants in appropriate amounts using the systems described above, the often large number of odourants that produce a specific aroma and the diversity of aromas is a major hurdle. As much as 10,000 odours can be detected by the human nose [14]; a simplistic aroma reconstruction system would thus consist of a mixing systems with 10,000 channels, each dispensing one particular odourant, which practically is not feasible. However, the total number of aroma receptor genes and corresponding receptor proteins that humans use to smell is relatively small (about 400 [15,16]). Nonetheless this allows us to discriminate between a vast universe of odours as the individual receptors are not very specific and respond to various degrees to different odourants [16]. This is similar to colour vision, where only three colour receptors respond to a different but broad and overlapping wavelength range of the electromagnetic spectrum allowing humans to perceive millions of colours. Inversely, in theory almost every imaginable colour can be blended using three primary colours only, although in practice usually four (cyan, magenta, yellow and black) are used in commercial printing applications. It, therefore, seems feasible to conceive odour primaries – mixtures of odourants with different but possibly partially overlapping

composition – that can reconstruct every imaginable smell [17]. While this is an exciting research question [18], it is beyond the scope of this manuscript which will be confined to the much smaller olfactory space generated by fruit odourants. Many aroma biosynthesis pathways are relatively well conserved amongst species and even beyond; the same odourants are often found in very different fruit, albeit in a different concentration and in combination with different other odourants. Within the *Citrus* genus, for example, the aroma is dominated by mostly the same terpenes and terpenoids but in different relatively concentrations [19]. Such confined aroma spaces are interesting because there are most likely no unique odourants per *Citrus* species; as a result trivial classifiers based on unique odourants can be excluded.

The hypothesis tested in this article is whether it would be possible to reconstruct fruit aromas by blending a relatively small number of odour primaries – essentially mixtures of pure odourants blended prior to the actual aroma synthesis. Nakamoto et al. [20] addressed this hypothesis before and attempted to reconstruct the aroma of 158 essential oils using 12 or 30 basis vectors using the non-negative least squares method. However, rather than the actual aroma composition, they reconstructed the mass spectral fingerprint. While fingerprinting based on mass spectra has been used successfully to discriminate odours [21], mass fragments are not unique, and pure odourant that evoke widely different odour perceptions may produce identical mass ions. For example, all monoterpenes have a molecular ion at m/z 136 and a typical fragmentation ion at m/z 93. Reconstructions with very similar mass spectrum but causing a completely different aroma perception are thus conceivable. A potentially better approach that we will investigate in this article is to derive primaries that allow to reconstruct the actual chemical composition of target aromas. We will adopt a data driven approach, using multivariate statistical techniques to project an aroma space onto a subspace of considerably lower dimension. The aroma reconstruction will be implemented in the latter subspace and aims to approximate the chemical composition of the target aromas rather than their overall mass spectrum as in [20]. To investigate this concept the relatively small aroma space spanned by *Citrus* species was targeted. Odour primaries were constructed and the original aroma space was projected on these odour primaries. Finally, the odour primaries were blended in real time in order to reconstruct the original aroma profiles and compare them later with the reference aroma profiles.

Materials and methods

Citrus samples

Five species of *Citrus* fruit were selected: 'Eureka' lemons (*Citrus* × *limon* (L.) Burm.f.) and 'Cambria' oranges (*Citrus* × *sinensis* (L.) Osbeck) from South-Africa, 'Tahiti' limes (*Citrus aurantifolia* (Christm.) Swingle), 'Ruby red' grapefruits (*Citrus* × *paradisi* Macfad.) from Mexico and 'Clementina Hoja' mandarins (*Citrus reticulata* Blanco) from Spain. The selection was based on commercial availability and diversity of their aromas. From each species, four different fruit were sampled for aroma analysis, resulting in a total of 20 samples. Fruit were cut to pieces, with the peels still attached as they contain most of the aroma compounds. These pieces were then mixed in a blender. Of each sample, 6.20 mL was diluted to 10 mL with 3.8 mL of a saturated KCl solution and mixed to stop the enzymatic reactions that would affect the aroma profile and to enhance the release of volatiles out of the matrix and into the headspace. All samples were collected in glass vials of 20 mL that were instantaneously frozen in liquid nitrogen and kept at -80°C until further analysis. The vials were sealed airtight by means of a cap with septum.

Aroma analyses

Each *Citrus* sample was thawed in a water bath at 35°C during 15 min, followed by incubation at 30°C during 1 hour to achieve equilibrium between the sample and its headspace. This was followed by an extraction step of 10 min using a StableFlex SPME fiber (df: 50/30 µm; measure: 24 gauge) coated with divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) from Supelco (Bornem, Belgium). The volatiles were desorbed from the fibre at 250°C into the a split/splitless injection port of an Agilent 6890N gas chromatograph (GC) (Agilent Technologies, Diegem, Belgium) coupled to an Agilent 5973 Network Mass Selective Detector (MS). The split ratio was set to 16:1 and the samples were injected by means of an autosampler (MPS2, Multipurpose sampler, Gerstel, Germany). The separation was achieved on a 5% phenylmethylsiloxane capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness, Supelco Co., Bellefonte, USA). Helium was used as carrier gas at a constant flow rate (1.24 mL min^{-1} at 30 °C). The GC temperature program was as follows: 55 °C (2 min), 85 °C (1.5 °C min^{-1}), 145 °C

(2.5 °C min^{-1}) and 240 °C (40 °C min^{-1}). The temperature was kept at 240°C for two more minutes.

Volatile compounds were identified through mass spectrometry with electron impact ionisation. Mass spectra were recorded at a scanning speed of 8 spectra s^{-1} over an m/z range of 10-350. The ionisation energy was 70 eV. Data were analysed using Chemstation software (G1701CA Version C.00.00, Agilent Technologies). The identification of volatile components was confirmed by comparison of collected mass spectra with those of commercial standards and spectra of the National Institute for Standards and Technology mass spectral library (NIST98, Search Version 2.0).

Data preprocessing

The total peak area per observation had a variance of $3.78 \cdot 10^{17}$ (arbitrary units) while the variance of the different odourants was between $2.48 \cdot 10^{11}$ (α-cubebene) and $4.96 \cdot 10^{17}$ (D-limonene). As this is a substantial difference, the peak area of each odourant was divided by the sum of the peak areas per observation to obtain a relative peak area. This removed any differences in total aroma production between samples. Note that, while peak area is proportional to concentration, this relation is different for all odourants. As not for all odourants that were identified standards were available, it was not possible to establish calibration curves to compute actual odourant concentrations except for those that were used to blend the primaries (see further).

Both the relative concentrations of odourants and their specific perception thresholds are responsible for perceived differences of the aroma of different species. Low concentrations of certain components may still dominate the overall aroma if they have a high abundance. Therefore, the peak areas of the odourants were weighted with their flavour dilution (FD) factor found in literature to create a first dataset ('FD weighed data') [22-26]. The flavour dilution factor essentially expresses how much an odourant can be diluted until it can no longer be perceived. Loosely speaking, the larger the FD of an odourant, the more intense its smell. For the odourants for which no FD factors could be found, the average value of their chemical class (e.g., monoterpenes) was used. This transformation ensured that components that had a low perception threshold (large FD factor) would obtain a higher weight in the further statistical analysis.

A second dataset ('FD weighed standardised data') was created by first dividing the relative peak area of each odourant by its standard deviation to avoid that odourants with a high abundance would dominate the analysis. Subsequently it was multiplied by the FD factor for reasons explained before.

Reduced order aroma space

The primaries could not be based on the loading weights of the components in the latent variables from any classical multivariate analysis such as PCA or PLS-DA as these coefficients can be negative; this would lead to negative concentrations that are physically impossible. A new approach was, therefore, developed. The problem is shown schematically in Fig. 1. The different *Citrus* species are denoted with the index i=1,…,n_{sp}; their corresponding aroma profile is $\mathbf{x}_i$ and this row vector contains the relative concentrations of the n_x selected pure odourants. The aroma profiles $\mathbf{x}_i$ can be stacked conveniently in a $n_{sp} \times n_x$ matrix **X**. The n_p primaries $\mathbf{p}_j$ are mixtures of the same n_x selected pure odourants and are stacked in a $n_P \times n_x$ matrix **P**, with

$$p_{j,k} \geq 0 \tag{1}$$

$$\sum_{k=1}^{n_x} p_{j,k} = 1, \quad \text{for all } j \tag{2}$$

Equation (1) ensures that the relative concentrations of the odourants in the primaries are positive, and equation (2) means that the relative concentrations of pure odourants in each primary add up to one. The objective is to synthesise the set of aroma profiles **X** by linearly combining n_P primaries $\mathbf{p}_j$

$$\mathbf{X} = \mathbf{AP} \tag{3}$$

with **A** an $(n_{sp} \times n_P)$ matrix that contains the channel coefficients $a_{i,j}$ that express how much of primary j should be taken to construct the aroma profile of species i. As the aroma profile is determined by relative amounts of odourants, all channel coefficients are expressed as fractions, and

$$a_{i,j} \geq 0 \tag{4}$$

$$\sum_{j=1}^{n_x} a_{i,j} = 1, \quad \text{for all } i \tag{5}$$

Equation (4) ensures that no nonsense negative amounts of a primary are to be added.

The coefficients of **A** and **P** were estimated by minimising the norm of the difference between reconstructed and measured aroma profiles $\widehat{\mathbf{X}}$ and **X** in a least square sense

while imposing the constraints in equations (1)-(5). The total number of parameters to be estimated was equal to $(n_P - 1) \times n_{sp} + (n_x - 1) \times n_P$. In the actual calculations, the coefficients of the first primary were fixed to the corresponding smallest value in all measured aroma profiles to stabilise the calculations. Note that the primaries calculated as described above are not necessarily orthogonal in contrast to, for example, the latent variables in a principal components analysis.

The nonlinear optimisation problem was implemented in Matlab 7 (The MathWorks Inc, Natick, MA), using the SSm GO toolbox from the Process Engineering Group IIM-CSIC in Vigo, Spain [27,28] that is based on a heuristic search algorithm that is robust with respect to local minima.

Selection of pure aroma volatiles

The pure aroma volatiles were selected based on a partial least squares discriminant analysis (PLS-DA) in combination with the Variables Important for Projection (VIP) [29].

PLS-DA essentially projects a data set with a large number of variables onto a smaller set of new latent variables, in such a way that observations that are characterised by a different value of a category variable are discriminated as much as possible [30-31]. The five *Citrus* species were described by means of five binary dummy variables. When an observation belonged to a particular species, the value of the corresponding dummy variable was set to 1; if not, the value was 0.

In order to reduce the number of pure odourants in the primaries, the least important components needed to be deleted from the datasets. This was done with the VIP procedure. For a given dimension, the VIP coefficient is equal to the weighed summation of the squared correlations between the LVs and the original variables. The weights are the fractions explained variability by that PLS dimension. In the normal (backward) VIP procedure, variables with the lowest VIP values, were deleted from the dataset. The procedure was then repeated until a list with a predefined number of key odourants was obtained [32-33]. This number was set to 15 to allow sufficiently complex aromas while at the same time limiting the number of pure odourants that in a further stage needed to be blended. We also implemented a forward VIP procedure, in which the odourant with the highest VIP coefficient was

selected. The VIP procedure was then repeated on the remaining data and in each iteration the odourant with the highest VIP coefficient was added to the list of key odourants. Both VIP methods were applied to both datasets, thus resulting in four lists of 15 key odourants. The discriminating power (total number of correct classifications of the 20 (5 species, 4 replicates) aroma profiles) of these four combinations of odourants was then compared and the one with the hightest value was chosen.

The Unscrambler software (Version 10.1, CAMO AS, Trondheim, Norway) was used for partial least squares-discriminant analysis (PLS-DA). The statistical models were validated by means of full cross validation (leave one out) because of the relatively low number of observations against the number of variables [34,35]

Aroma synthesiser

An aroma synthesiser was constructed with its body consisting of two hollow metal hemispheres of stainless steel (diameter 36 cm) joined together by means of a wooden frame, together forming a complete sphere (Fig. 2). In the top hemisphere, four nozzles (nozzle diameter 0.18 mm; 70-410 kPa; 0.5 mL reservoir; airbrush P 410, Conrad, Mortsel, Belgium) were positioned pointing towards the centre, each connected to a computer controlled valve (PV221-24VDC-1/8 type, RoboPos, Waalwijk, The Netherlands) that supplied nitrogen gas from a pressure vessel at 12.5 kPa via an electronic pressure controller. An Arduino Mega 2560 microcontroller board was used to control the setup by means of a C++ program with a windows interface written in Windev 7 (PC Soft, Montpellier, France). Communication was done through a serial connection. The control software allowed to set different spraying times for each of the four nozzles and to flush all nozzles simultaneously with nitrogen gas.

The 12 (see further) odourants used were D-limonene, α-pinene, β-pinene, linalool, octanal, nerylacetate, α-terpinene, β-myrcene (all from Sigma Aldrich, Bornem, Belgium), γ-terpinene, α-terpineol (both from Acros, Geel, Belgium), terpinolene (TCI, Zwijndrecht, Belgium) and geranial (synonym for citral A) from Alfa Aesar (Karlsruhe, Germany). The odourants were dissolved in isopropanol (Sigma Aldrich) that has a high odour threshold. The nozzles were all set to roughly the same flow rate (± 55 µL s^{-1}). This was checked by measuring the time needed to dispense a known volume of isopropanol. Between each spraying, the sphere was cleansed with

a paper cloth impregnated with isopropanol and left open under the hood for any remaining volatiles to evaporate.

To calibrate the aroma synthesiser, a test mixture was used consisting of a reduced list of 8 odourants dissolved in isopropanol to various concentrations: 10 mM α-pinene, 1 mM β-pinene, 1 mM octanal, 100 mM D-limonene, 1 mM γ-terpinene, 1 mM terpinolene, 1 mM linalool and 1 mM α-terpineol. The mixture was put in each channel and dispensed for different times, ranging between 0.25 and 3 s. At the top of the sphere, an SPME fibre was inserted immediately after the spraying to extract the volatilised odourants from the sphere's space. After an absorption time of 10 min, the fibre was removed from the sphere and inserted in the GC-MS for desorption and analysis of the components. Preliminary tests showed that only during the first 2 s of spraying a linear relationship between spraying time and peak surface was found, presumably because of saturation of the SPME fibre. This time was, therefore, taken as the maximum time for the nozzles. A linear relationship between spraying time, up to 2 s, and peak area was obtained for each of the 8 odourants. The coefficient of variation of the peak areas was 10.2% on average. Next, the test mixture was diluted to 25, 50 and 75% in isopropanol and sprayed for 2 s by every nozzle. Also this relation proved to be linear with an average standard deviation of 9.9% of the measured peak areas. As the response proved to be linear, measuring one point on the graphs was enough to calibrate the setup for the four remaining odourants.

Validation of the reconstructed aroma profiles

The analysis of the reconstructed fruit aromas was done on a fast GC-MS. This was an identical GC-MS system to the one used for the *Citrus* aroma analyses, but combined with a modular accelerated column heater (MACH) system (Gerstel, Germany) in which the column was mounted. Aroma compounds were thermally desorbed from the fibre into a split/splitless injector heated at 250 °C at a ratio of 50:1. Separation was done on a DB1-MS capillary column (10 m x 0.1 mm internal diameter, 0.4 µm film thickness). Helium was used as carrier gas under constant pressure (600 kPa - 0.7 mL min^{-1} at 30 °C). The GC temperature program was as follows: 30 °C (1 min), 110 °C (100 °C min^{-1}), 190 °C (10 °C min^{-1}) and 250 °C (100 °C min^{-1}). The temperature was kept at 250°C for one more minute.. Mass spectra in the 35 to 315 m/z range were recorded at a scanning speed of 8.99 scans s^{-1}. The

chromatography and spectral data were evaluated using MSD ChemStation Software (Agilent Technologies, USA) and AMDIS v.2.1 (NIST, USA). Volatile compounds were identified with the NIST98 database (NIST98 v.2.0, USA) and by retention indices.

Results

Citrus aroma analysis

In total 45 volatile components were identified of which D-limonene was the main component for every species (Table 1). Not all 45 components were present in every species. Lemon and lime had relatively more components: 31 and 35 respectively, compared to 25 for orange and mandarin, and 26 for grapefruit. The components neral, α-bergamotene, α-bisabolene, nerylacetate, α-terpinene and geranylacetate were exclusively present in lemon and lime. On the other hand, E-2-hexenal was not present in the aroma of lime and lemon, but contributed considerably to the composition of the volatile profile of the other three species. Furthermore, there were some compounds that were only present in 1 single species: 1-octanol in orange, β- and δ-elemene in lime and α-cubebene in grapefruit.

Fig. 3A shows the score plot of the PLS-DA for LV4 versus LV1 based on the weighted dataset. The explained X-variance was 99.99% for the first four LVs. It is clear that all species are discriminated well, which was not the case for the score plots of LV2 vs LV1, LV3 vs LV1 or LV3 vs LV2. The corresponding correlation loading plot (Fig. 3B) reveals a clear set of relevant volatile components, mainly oriented along the LV1 axis (e.g., D-limonene, β-myrcene, β-pinene, γ-terpinene, α-pinene) and to a lesser extent along the LV4 axis (e.g., 1-octanol, valencene).

A discrimination model was established to classify the samples. All samples were classified in the right category, but the predicted value of the mandarin dummy variable of one mandarin sample (Ma 1, Fig. 3A) was closer to 0 than to 1 indicating a high degree of agreement with the other categories as well. Still, it was classified correctly. The number of observations was too small for defining a validation test set.

A slightly lower classification performance (95%) was obtained for the FD weighted standardised dataset (results not shown).

Selection of pure volatile components

By applying the forward VIP to the FD weighted dataset a correct classification percentage of 95% could be maintained while in the other approaches it dropped to 80% and below. The R^2 of the PLS-DA models per *Citrus* species were on average better for the forward VIP on the FD weighed dataset except for lime and lemon for which there was hardly any difference between the approaches (Fig 4). For grapefruit in particular the forward VIP outperformed the backward VIP considerably.

The 15 most significant variables that were identified through the forward VIP applied to the FD weigthed dataset are printed in bold face in Table 1. Almost identical components were identified by the different approaches: for the backward VIP, the same components were retained for both datasets; 4 components were different between the two forward VIPs. Based on these results, the forward VIP on the FD weighted dataset was chosen for further use. As no commercial supplier of neral, α-thujene and β-phellandrene was found, these components were removed from the list. Camphene was also excluded as it crystallised and clogged the nozzles of the aroma synthesiser during spraying. To compensate for this, the next component of the list, octanal, was included. This resulted in a 100% correct classification accuracy, although the R^2 values for some fruit species were rather low (0.94; 0.41; 0.88; 0.44; 0.69 for lime, orange, lemon, mandarin and grapefruit, respectively). The small effect of the deletion of 'higher ranked' components indicate that it is not the presence of specific single components playing a major role, but the ratio of some key components.

Aroma reconstruction

The algorithm for aroma reconstruction was executed for 1 up to 5 primaries. The residual error decreased rapidly with an increasing number of channels, and with 5 channels a theoretical classification efficiency of 100% was obtained. With 4 channels, the classification efficiency was 80%: grapefruit was wrongly classified as orange. The score plot for LV4 versus LV1 of this set is shown in Fig. 5. Note that although mandarin and orange were not discriminated well in this plot, they were in the score plot of LV3 versus LV1 (not shown). The model parameters are listed in Table 2.

Subsequently, the mixtures for all primaries were assembled. The theoretically calculated fraction of D-limonene in primary 1 was the highest of all components in all primaries (= 0.9530 in Table 2). The actual concentration of the aroma components in every primary was, therefore, calculated relative to that of limonene (100 mM, 2 s spraying time) using the values of $p_{i,j}$ in table 2 and using the previously established calibration curves. To validate their actual composition in the air, these primaries were sprayed for 2 s by their corresponding nozzle. As an illustration, the resulting normalised aroma profile of primary 2, as captured by the SPME fibre, is compared to the normalised theoretical compositions in Fig. 6. Very similar results were obtained for the other primaries. The channel coefficients were also adjusted in such a way that, for every fruit species, the highest coefficient corresponded to a spraying time of 2 s, and are listed in Table 2. The spraying times of the other three channels were calculated relative to this. The final spraying times in milliseconds are shown in Table 3. The lowest spraying time was 21 ms. When the calculated spraying time was lower than this, the channel was kept closed.

To synthesise the aroma of a fruit species, the primaries were loaded in all nozzles and sprayed according to the respective spraying times for that fruit species. As an illustration, the normalised reconstructed aroma profile of lime is compared to the normalised reference profile in Fig. 7. All reconstructed aroma profiles (n = 3 per aroma) were close to the reference aromas, with a standard deviation of 18.2% of the actual component peak areas on average. This is higher than the deviation for each nozzle separately (= 9.3%).

The measured aromas were also used to test the discrimination model that was derived before. The score plot of LV1-LV4 is shown in Fig. 8. Lime and lemon were classified correctly in all replicates. For orange, two out of three samples of the reproduced aromas (66.7%) were classified correctly and one sample was misclassified as mandarin. Similar results were obtained for mandarin (66.7%) with one of three samples being misclassified as orange. Finally, only one grapefruit aroma was correctly classified and two misclassified as mandarin (33.3%). The overall correct classification percentage was 73%.

Discussion

Citrus species can be discriminated based on their headspace volatile composition

Because of the fragmented information that is available and also the wide variety of aroma isolation methods, SPME fibre composition, GC and MS settings, it is only possible to qualitatively compare the aroma profiles with literature results. All identified odourants were found earlier in the headspace or essential oil of *Citrus* species [36-41]. Many odourants were shared amongst the species. Those odourants that from our analyses appeared to be unique to a species have been reported before in the headspace or essential oil of other *Citrus* species; for example, cubebene (here unique to grapefruit) has been found in orange [42], octanol (unique to orange) also in mandarin [37], and β-elemene (unique to lime) also in orange [44]. This suggests that the biosynthesis pathways of odourants are conserved in the *Citrus* genus, but their activity is different in the different species.

Using a PLS-DA, all species could be discriminated based on their aroma profile. However, this discrimination was only based on the chemical composition of the headspace and did not incorporate sensory information except indirectly through the flavour dilution factors of the odourants that were incorporated in the dataset. It is very well possible that the actual olfactory differences between the aroma of different species was due to trace components, possibly even beyond the detection limit of our GC-MS system. Some typical odourants of *Citrus* were not found indeed, including ethyl butanoate in orange [42] and nootkatone in grapefruit [43].

Aroma synthesiser allows blending of aromas in real time

The aroma synthesiser was constructed using low cost components such as a stainless steel sphere with four consumer grade air brush nozzles controlled by an Arduino unit. As the nozzles were the most expensive part of the synthesiser, it was important to limit the number of primaries as much as possible. Note that the synthesiser only allowed blending primaries for testing purposes but did not have special amenities for distributing the synthesised aroma into the ambient space and directing the synthesised aroma to an end-user.

The minimum and maximum spraying time were set to 20-2000 ms, as in this range a linear relationship between headspace concentration (as measured by peak area in the total ion chromatogram) of the odourants and spraying time was obtained, as well as between headspace concentration and concentration in the primary solution. This

indicates that the setup was useful to dispense primaries in a controlled and quantitative way.

As the mean diffusion coefficient *D* of D-limonene in air has been reported to be equal to 5.64×10^{-6} m^2/s [45], the root mean square distance a molecule has travelled in *t* = 2000 ms is equal to $< x >= \sqrt{2Dt} = 4.7$ mm. Even if turbulent dispersion would increase the diffusion (dispersion) coefficient a thousand fold, it would take more time for the odourants to travel from an outlet of the aroma synthesiser to the aroma receptors in the nose than to actually synthesize the aroma. As the time constant of the blending process is thus small compared to that of the olfaction process, real time aroma synthesis is thus possible.

Aroma reconstruction using a limited number of primaries had an accuracy of 73%

The main objective of this article was to evaluate whether odours can be reconstructed in real time by mixing a relatively small amount of odour primaries being a mixture of pure odourants. To this purpose we constructed odour primaries as a (not necessarily orthogonal) basis of an odour space of reduced dimensionality so as to maximise the discrimination of five species of *Citrus* fruit. Four primaries composed of 13 different pure odourants were used to obtain an overall theoretical classification accuracy of 80% which largely verified our hypothesis. The actual classification accuracy achieved within the aroma synthesiser equalled to 73%. The imperfect classification was likely due to the close similarity of the aroma composition of orange, mandarin and grapefruit and the inaccuracy of the nozzles. Tamura et al. [46] used 11 volatiles to reconstruct the aroma of orange juice, of which five were also used in our list of odourants that was used to construct the primaries. Buettner and Schieberle [47] reconstructed the aroma of orange juice using 22 odourants based on aroma extract dilution analysis (AEDA) in combination with GC olfactometry. Five of them were also included in our list. Petersen et al. [48] used PLS to identify eleven key volatiles in the headspace of orange aroma, of which four were in common with our list. While there is a clear overlap between our list of key odourants and the reported lists, any differences may partially be due to the fact that our list was established with the aim of reconstructing the aroma of five *Citrus* species instead of one. Nakamoto et al. [19] derived primaries for an aroma space spanned by 158 commercial essential oils of a range of plant species. They reconstructed three aromas and used a consumer panel to

classify them. When 30 primaries were used most panellists correctly classified both the target and reconstructed aromas; when 12 primaries were used the classification was worse.

An alternative approach would be to start from sensory descriptors instead of odourants. Instead of primaries consisting of mixtures of pure odourants, the aroma space would be spanned by primaries based on sensory descriptors, and similar techniques as the one described in this article could be used to reduce its dimension. Dimensionalities as low as 6 [49], 9 [50], 10 [18] up to 32-64 [17] have been reported. Mamlouk and Martinetz [17] statistically analysed a large database of odourants and their corresponding olfactory perceptions. They found that the original set of 171 descriptors (after removing descriptors that were evoked by a single chemical) could be reduced to 64 and even 32 with minimal loss of information. A similar approach based on non-negative matrix factorization has been followed by Castro et al. [18]. For aroma synthesis, it is conceivable that the latent variables that would be obtained through such approaches could be translated in an additional step into primaries consisting of one or more odourants that subsequently could be loaded into the different channels of the aroma synthesiser. Our results show that even with 4 primaries and 13 different pure odourants it was difficult to discriminate aromas of five *Citrus* species. This is rather surprising, as with five primaries a perfect (but trivial) reconstruction would be possible by loading the essential oils of the five considered species in the channels. While no sensory analysis was performed in this work, one would expect that a human expert would be capable of discriminating these species easily. It is conceivable that the relatively low odour space dimensionalities based on sensory descriptors that were mentioned in the aforementioned references rather illustrate the inability of the English vocabulary to describe aroma in all its subtleties. Indeed, many odourants that have been observed in the aroma of *Citrus* species are described as having a ‘citrus-like’ aroma. Further, if the real dimension of the odour space would indeed be much smaller than the number of different aroma receptors and their corresponding genes (a few hundreds), one might speculate that their number would have decreased correspondingly in the course of evolution.

Conclusions

A procedure was developed to derive odour primaries as (nonorthogonal) basis functions that span a (reduced) aroma space of interest. The odour primaries are mixtures of pure aroma components and can be blended to reconstruct arbitrary aromas within the original aroma space. To investigate the concept, the aromas of five *Citrus* species (lime, lemon, orange, grapefruit and mandarin) were analysed by means of GC-MS, the aroma primaries were computed and blended off-line using 12 pure odourants. These primaries were loaded into the four nozzles of a purpose built aroma synthesiser to spray and mix the aromas of the five *Citrus* species in real time. Overall the classification accuracy of the reconstructed aromas was 73 %. It is necessary to emphasize that, while for practical reasons we used GC-MS in this manuscript to compare the reconstructed citrus aromas with the original ones, in future research these results need to be validated by human sensory panels.

The results indicated that the construction of an aroma synthesiser to reconstruct real aromas is feasible, but the number of primaries and also pure odourants to construct these primaries was higher than anticipated and the accuracy was not perfect. Further experiments are required to test the procedure on aroma spaces of higher dimensionalities and to evaluate whether the errors associated with spraying the odourants are small enough to allow for reliable reconstruction of unique aromas. Also, alternative approaches based on decomposition of aroma spaces constructed using sensory descriptors and subsequent translation into mixtures of pure odourants need to be investigated further.

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Bart Nicolai obtained a MSc in bioscience engineering from Ghent University (Belgium), and a MSc in Applied Mathematics and PhD in Bioscience Engineering from the University of Leuven (Belgium). He is now full professor at the latter institute and head of the Biosystems Department. In addition, he is responsible for co-ordinating the research in the Flanders Center of Postharvest Technology (VCBT), an experimental facility which was established as a public–private partnership between the University of Leuven and the Association of Belgian Horticultural Co-operations in 1997. His main research interests are postharvest biology and technology, heat and mass transfer, and fruit and vegetable quality with flavor in particular. He is associate editor of the journal Postharvest Biology and Technology.

Evelien Micholt holds a MSc in Bioscience Engineering from the University of Leuven (Belgium). For her master thesis, she focused on odorant delivery systems, varying from spraying devices to piezoelectric dispensing systems. She obtained a PhD on cell based bioelectronics noses in a joint project of the MeBioS division of the Biosystems Department at the University of Leuven and the Bioelectronic Systems group of IMEC (Leuven). She is now Functional Designer at Nobel Biocare.

Nico Scheerlinck has a MSc in Agricultural Engineering, a PhD in Bioscience Engineering, and an academic teaching qualification in mathematics from the University of Leuven (Belgium). During his PhD research and postdoctoral fellowships at the University of Oxford (UK) and Purdue University (US) he carried out research in applied, mathematical and computational biology, with special emphasis on applications in food technology, and postharvest biology and technology. His main expertise is in applying mathematical/computational methodologies for investigating biological mechanisms, processes and systems. He is now a teacher and tutor in mathematical engineering at the University of Leuven.

Thomas Vandendriessche obtained a MSc in biology at the University of Leuven (Belgium) in 2005. In 2011 he obtained a PhD in Pharmaceutical Sciences at the Lab of Toxicology, University of Leuven. During this PhD, he mainly focused on the purification, structural and functional characterization of novel voltage-gated ion channel ligands from amphibians and scorpions. In 2012 he finalised a PhD in Bioscience Engineering at the Division MeBioS (Mechatronics, Biostatistics and Sensors), University of Leuven. During this PhD he developed and implemented fast techniques for analysing strawberry aroma. In 2013, he

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Maarten Hertog obtained his MSc in Biology at the University of Utrecht (NL) and obtained his PhD in the Applied Biological Sciences at KU Leuven (BE). As research manager computational plant biology at KU Leuven he is responsible for various applied system biology projects in the area of postharvest biology integrating the various research disciplines operating at the various control levels through kinetic modeling approaches. He is editor of four books in the area of food modeling and is member of the editorial board of Postharvest Biology and Technology.

Ian Ferguson has a BSc (Hons) and a PhD from the University of Auckland (New Zealand). He is past Chief Science Advisor at Plant & Food Research (New Zealand) and Departmental Science Advisor of the New Zealand Ministry of Primary Industries. He specialises in postharvest science, plant and fruit physiology, biochemistry and biotechnology, particularly in postharvest physiology of disorders and stress responses, fruit genomics, and systems approaches. He is honorary editor of the international journal Postharvest Biology & Technology. He is also Visiting Professor, College of Life Sciences, Zhejiang University, Hangzhou, PR China.

Jeroen Lammertyn holds a MSc in Bioscience Engineering and a MSc in Biostatistics. He obtained his PhD in Bioscience Engineering at the University of Leuven in 2001. From 2002 to 2005 he worked as postdoctoral researcher and spent one year as a research associate at the Pennsylvania State University, USA. Since 2005 he is appointed Professor at the University of Leuven. His main research interests involve bionanotechnology and more specifically biosensor development, bionanohybrid materials, micro- and nanofluidics and bio-assay development. He teaches in the Master programs of Bioscience Engineering and Nanoscience and Nanotechnology. He is author or co-author of 100+ peer reviewed research papers and over 120 conference papers, and acts as reviewer for many international journals.

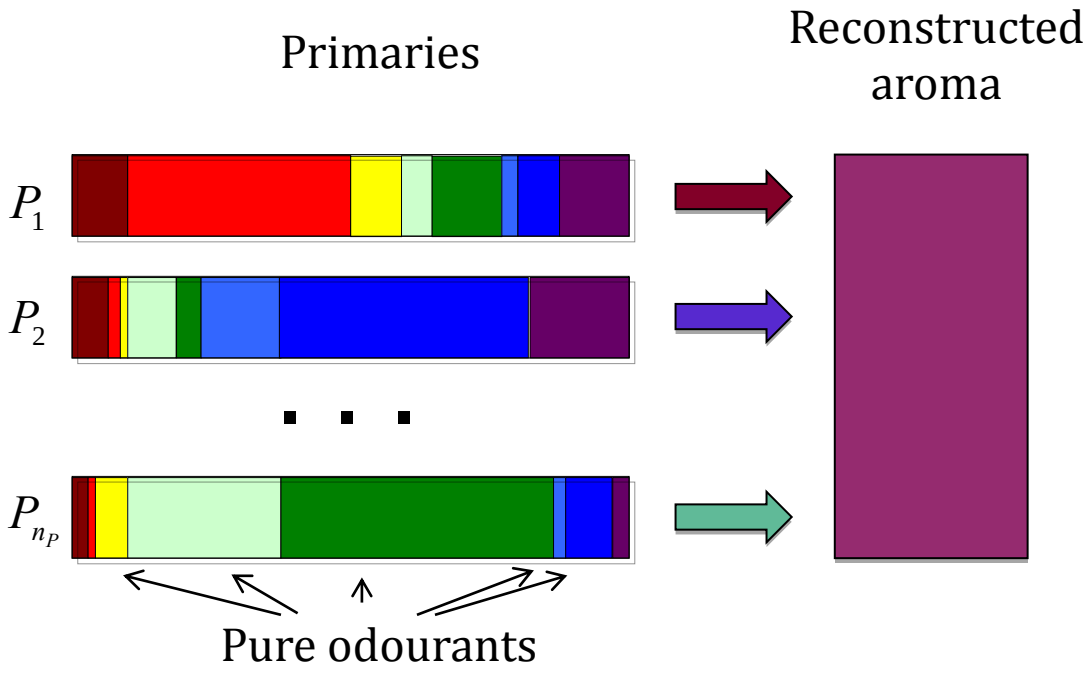
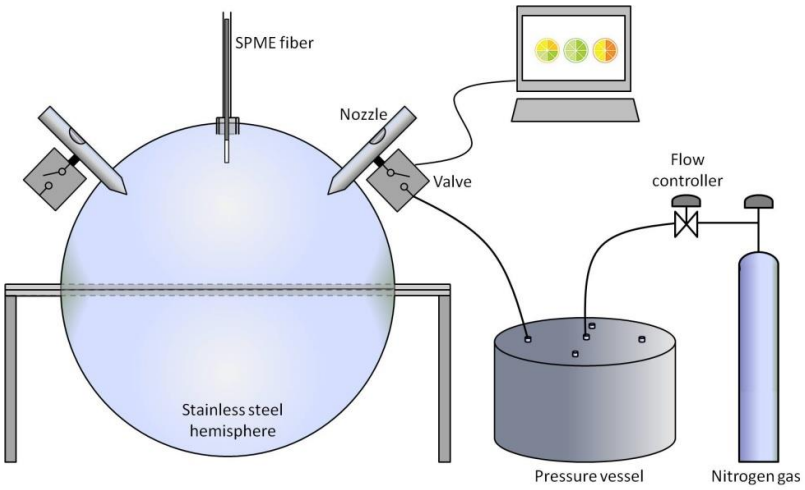


Figure 1. Principle of the aroma synthesiser. Pure odourants are mixed off-line to obtain so-called primaries that are then loaded into dispensing units. The latter spray well-defined amounts of primaries into an enclosure where they evaporate and blend. The composition of the primaries is selected before in such a way that the reconstructed aromas optimally match the reference aromas.

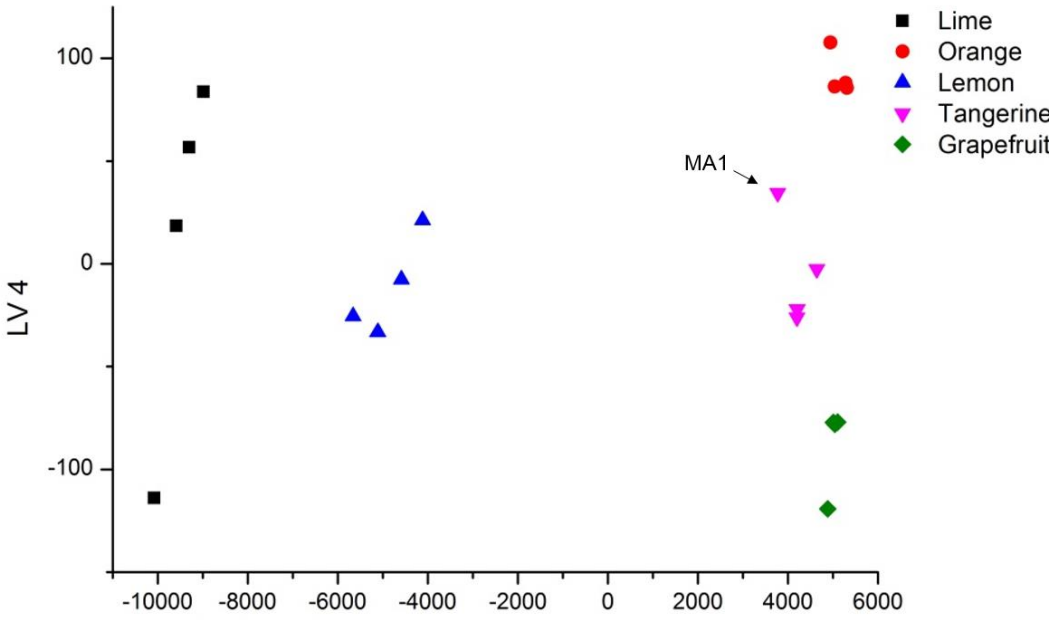
A.



B.

Figure 2. Schematic (A) and photograph (B) of the two hemispheres and the wooden frame of the aroma synthesiser. Pressurised air is guided via a pressure vessel towards the nozzles that spray the primaries into the spherical enclosure. The SPME fibre is inserted through a hole at the top after the pure odourants in the sprayed primaries are evaporated.

A.



B.

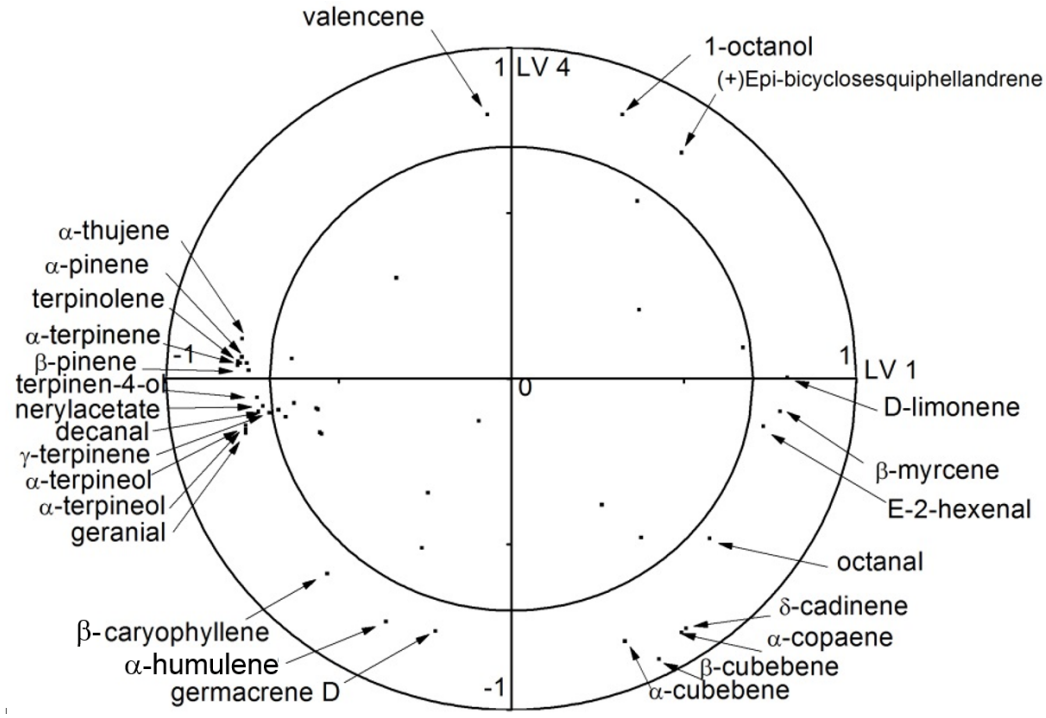


Figure 3. PLS-DA score (A) and correlation loading (B) plot of the FD weighted data: LV4 versus LV1. The label 'MA1' indicates the outlier sample in the PLS-DA classification (see text). Variables within the inner circle of the correlation loading plot explain less than 50% of the variation in the data and were not further considered.

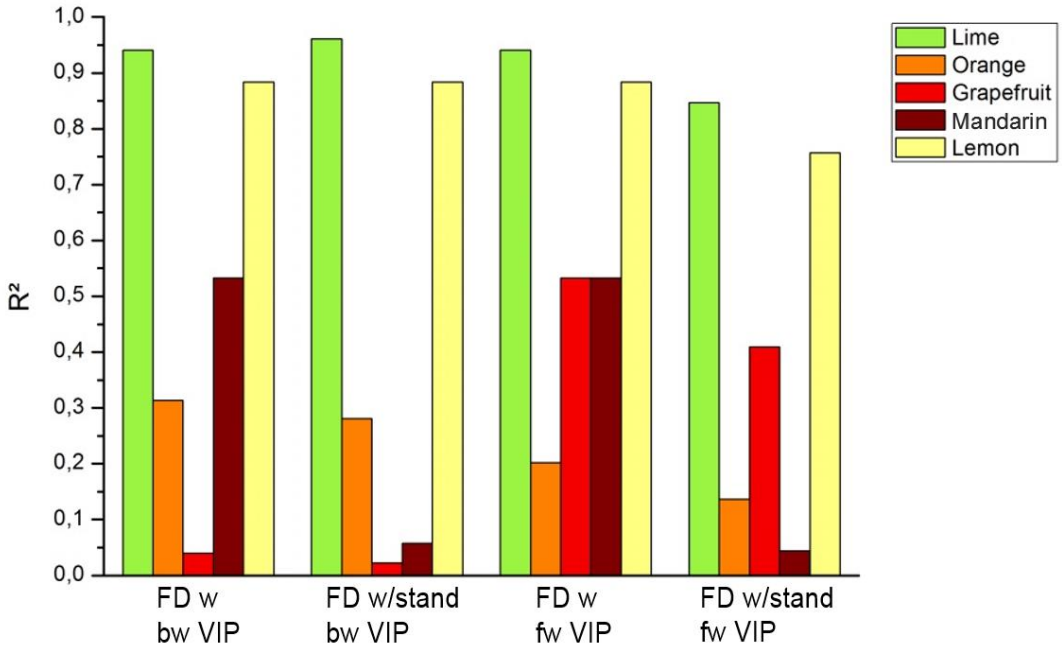


Figure 4. R^2 of PLS-DA models to discriminate five citrus species based on 15 components obtained through a forward (fw) and backward (b) VIP analysis on the FD weighted (FD w) and FD weighted standardised (FD w/stand) datasets.

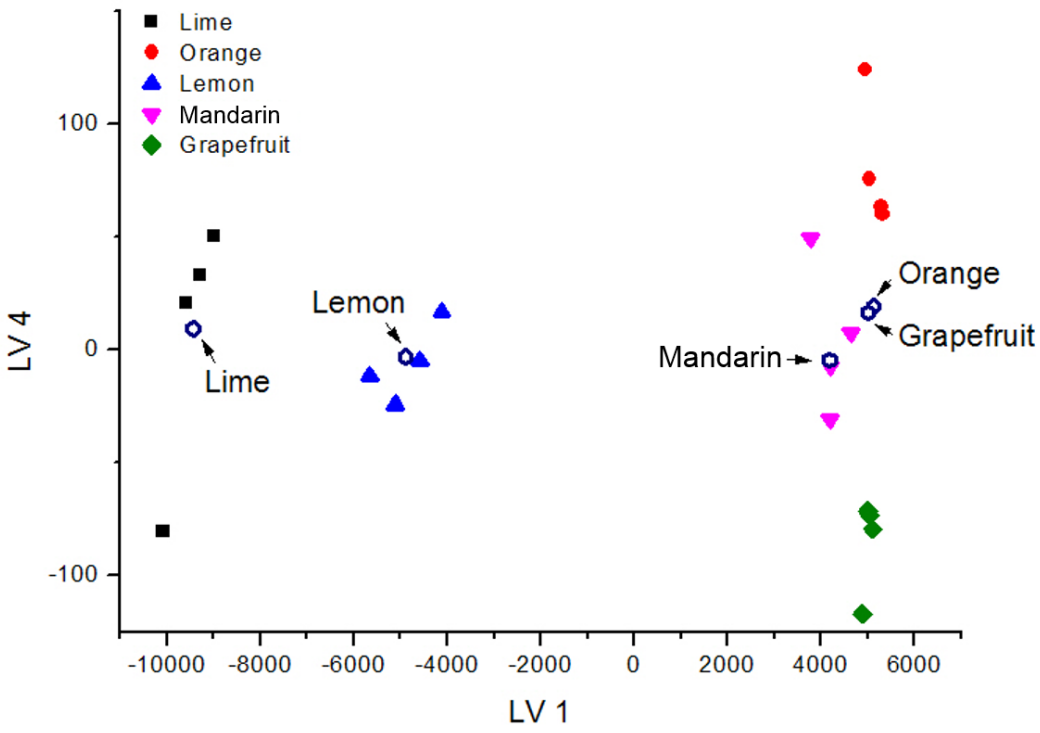


Figure 5. PLS-DA score plot (LV4 versus LV1) of the reduced aroma profiles of five citrus species obtained with four primaries.

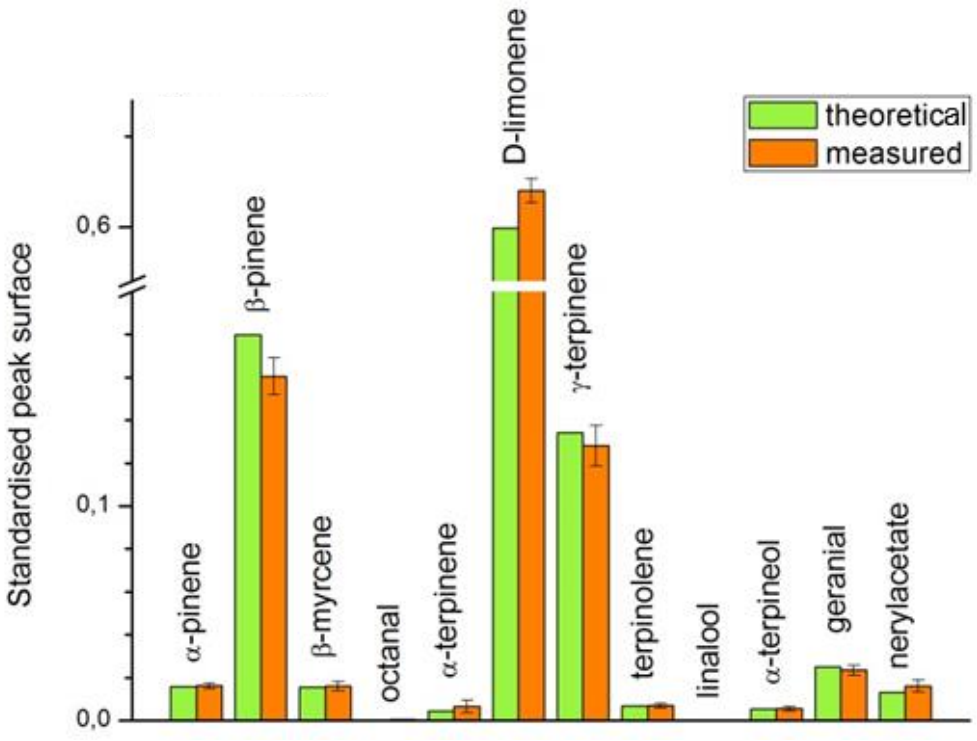


Figure 6. Normalised theoretical and mean measured peak areas of the aroma profile of primary 2. Error bars are standard deviations, n = 3.

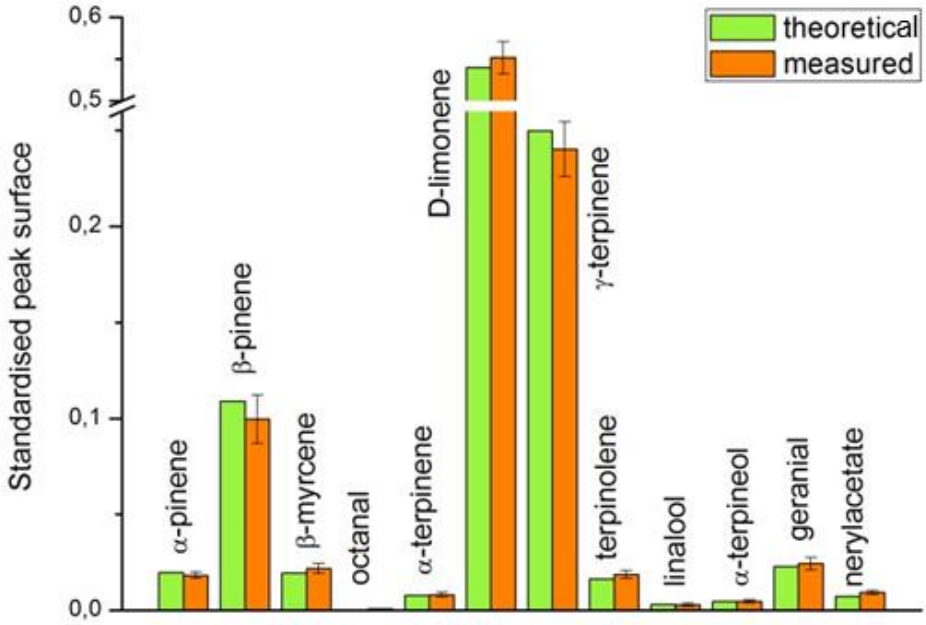


Figure 7. PLS-DA score plot (LV4 versus LV1) of the original and reconstructed lime aroma.

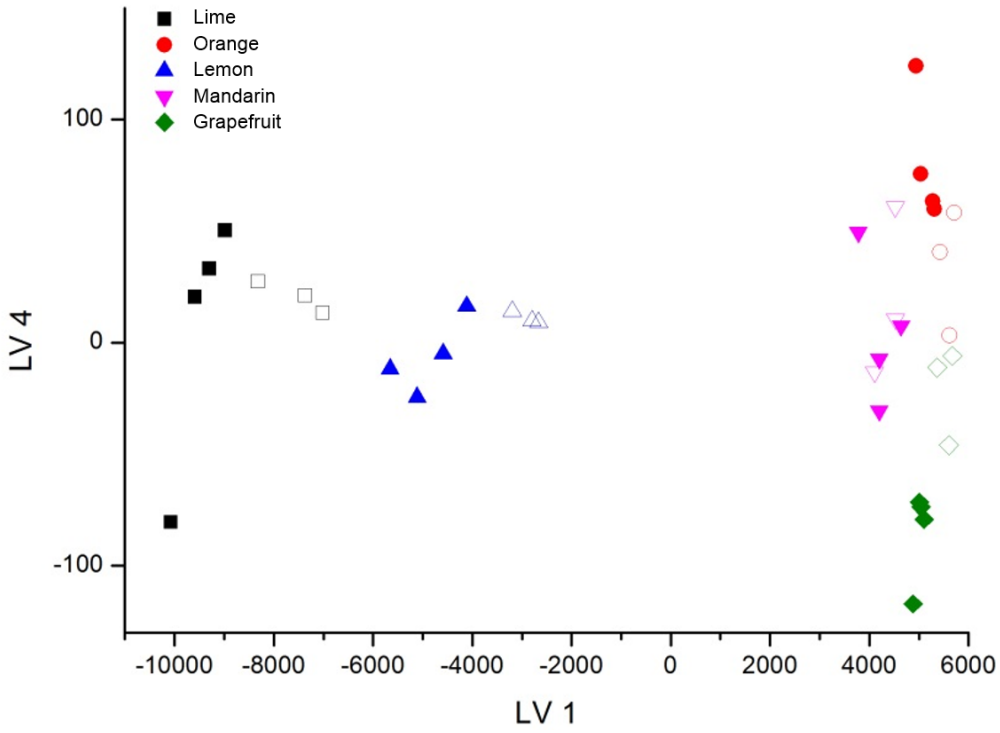


Figure 8. PLS-DA score plot (LV4 versus LV1) of the reconstructed (open symbols) and measured (closed symbols) citrus aromas.

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Table 1. Retention times and relative measured peak areas of all measured odourants of the five citrus fruits. The odourants selected by the forward VIP procedure on the FD weighted dataset are in bold face. Odourants for which no references were available were tentatively (t) identified based on their mass spectrum and retention index.

					Mean relative peak areas (%)				
	Odorant	**Group**	**Retention time (min)**	**Mean log_3(FD)(*)**	**Lime**	**Orange**	**Lemon**	**Mandarin**	**Grapefruit**
1	E-2-Hexenal	aldehyde	4.41	5.07 (**)	0.00	0.14	0.00	0.13	0.16
2	**α-Thujene** (t)	monoterpene	6.85	5.13 (**)	0.60	0.02	0.33	0.05	0.00
3	**1S-α-Pinene**	monoterpene	7.1	5.83	1.75	0.50	1.38	0.57	0.54
4	**Camphene**	monoterpene	7.67	3.83	0.07	0.00	0.06	0.00	0.00
5	**β-Phellandrene** (t)	monoterpene	8.77	4.80	1.77	1.03	2.02	2.33	0.32
6	**β-Pinene**	monoterpene	8.95	6.00	9.73	0.06	10.45	0.13	0.04
7	**β-Myrcene**	monoterpene	9.72	6.50	1.73	2.78	1.93	2.94	2.99
8	Octanal	aldehyde	10.16	5.00	0.04	0.25	0.08	0.95	0.75
9	α- Phellandrene (t)	monoterpene	10.33	4.60	0.10	0.09	0.07	0.11	0.12
10	3-Carene (t)	monoterpene	10.67	5.13 (**)	0.00	0.06	0.00	0.17	0.00

11	**α-Terpinene**	monoterpene	11.01	4.50	0.71	0.00	0.39	0.00	0.00
12	**D-Limonene**	monoterpene	11.9	6.83	48.75	93.00	62.87	89.95	92.55
13	Trans-β-ocimene t)	monoterpene	12.41	4.00	0.07	0.00	0.10	0.00	0.00
14	Cis-ocimene (t)	monoterpene	13	4.33	0.18	0.05	0.25	0.21	0.19
15	**γ-Terpinene**	monoterpene	13.59	5.20	22.74	0.04	12.40	0.11	0.04
16	1-octanol	alcohol	14.49	4.00	0.00	0.11	0.00	0.00	0.00
17	**Terpinolene**	monoterpene	15.45	3.83	1.48	0.04	0.74	0.08	0.03
18	**Linalool**	monoterpene alcohol	16.32	6.50	0.29	0.69	0.21	0.95	0.19
19	Nonanal	aldehyde	16.51	5.00	0.02	0.11	0.24	0.00	0.11
20	Citronellal	monoterpene aldehyde	20.06	5.40	0.00	0.00	0.00	0.10	0.05
21	Terpinen-4-ol	monoterpene alcohol	21.7	4.20	0.48	0.02	0.58	0.08	0.01
22	**α-Terpineol**	monoterpene alcohol	22.76	4.80	0.42	0.04	0.37	0.10	0.06
23	Decanal	aldehyde	24.08	5.20	0.25	0.66	0.07	0.77	0.58
24	**Neral** (t)	monoterpene aldehyde	26.4	4.00	1.49	0.00	1.13	0.00	0.00
25	**Geranial**	monoterpene aldehyde	28.4	5.67	2.10	0.03	1.65	0.00	0.06
26	δ-Elemene (t)	sesquiterpene	32.64	5.50	0.08	0.00	0.00	0.00	0.00

27	α-Cubebene (t)	sesquiterpene	33.35	1.00	0.00	0.00	0.00	0.00	0.04
28	**Nerylacetate**	monoterpene ester	34.3	3.67	0.71	0.00	0.75	0.00	0.00
29	α-Copaene (t)	sesquiterpene	34.75	5.00	0.00	0.03	0.00	0.07	0.21
30	Geranylacetate (t)	monoterpene ester	35.38	3.50	0.19	0.00	0.35	0.00	0.00
31	β-Cubebene (t)	sesquiterpene	35.58	5.00	0.00	0.00	0.00	0.04	0.12
32	β-Elemene (t)	sesquiterpene	35.71	4.80	0.10	0.00	0.00	0.00	0.00
33	β-Caryophyllene (t)	sesquiterpene	37.02	4.00	0.80	0.04	0.34	0.02	0.53
34	(+)Epi-bicyclosesqui-phellandrene (t)	sesquiterpene	37.6	4.26 (**)	0.00	0.05	0.00	0.04	0.00
35	α-Bergamotene (t)	sesquiterpene	38.12	3.00	1.35	0.00	0.49	0.00	0.00
36	α-humulene (t)	sesquiterpene	38.8	4.00	0.06	0.00	0.02	0.00	0.06
37	β-Farnesene (t)	sesquiterpene	39.34	4.50	0.10	0.00	0.02	0.00	0.00
38	Germacrene D (t)	sesquiterpene	40.24	5.00	0.07	0.00	0.00	0.02	0.06
39	Valencene (t)	sesquiterpene	40.89	4.33	0.05	0.14	0.07	0.00	0.00
40	γ-Elemene (t)	sesquiterpen	41.05	4.00	0.00	0.00	0.15	0.00	0.04

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41	Germacrene A (t)	sesquiterpene	41.44	4.26 (**)	0.10	0.00	0.00	0.00	0.00
42	α-Bisabolene (t)	sesquiterpene	41.58	4.26 (**)	0.11	0.00	0.03	0.00	0.00
43	β-Bisabolene (t)	sesquiterpene	41.86	4.26 (**)	1.35	0.00	0.45	0.04	0.00
44	δ-Cadinene (t)	sesquiterpene	42.47	5.00	0.00	0.02	0.00	0.05	0.13
45	Germacrene B (t)	sesquiterpene	43.92	4.50	0.14	0.00	0.00	0.00	0.00

(*) Minh Tu *et al.*, 2002; Sawamura *et al.*, 2004; 2006; Choi *et al.*, 2002;2005; Phi *et al.*, 2006

(**) calculated as the mean FD factor of the group to which the component belongs

Table 2. Estimated values of $a_{i,j}$ and $p_{j,k}$ with *i*, *j* and *k* denoting species, primary and odorant, respectively (see text).

		Primary 1	**Primary 2**	**Primary 3**	**Primary 4**
$a_{i,j}$	**Lime**	5.6012	15.7435	19.0641	0.0894
	Orange	24.5068	0.0283	0.0004	13.1210
	Lemon	8.0017	18.9019	4.5930	2.1119
	Mandarin	5.7721	0.2091	0.0416	32.9810
	Grapefruit	17.9260	0.0204	0.0037	14.1297
$p_{j,k}$	**α-Pinene**	0.0090	0.0159	0.0262	0.0040
	β-Pinene	0.0005	0.1799	0.0829	0.0003
	β-Myrcene	0.0319	0.0155	0.0188	0.0294
	Octanal	0.0008	0.0002	0.0002	0.0116
	α-Terpinene	0.0000	0.0044	0.0131	0.0000
	D-Limonene	0.9530	0.5994	0.3669	0.9426
	γ-Terpinene	0.0004	0.1342	0.4197	0.0001
	Terpinolene	0.0006	0.0068	0.0290	0.0006
	Linalool	0.0031	0.0002	0.0054	0.0103
	α-Terpineol	0.0006	0.0053	0.0052	0.0009
	Geranial	0.0000	0.0251	0.0277	0.0001
	Nerylacetate	0.0000	0.0131	0.0048	0.0001

Table 3. Nozzle spraying times (ms) per fruit species.

	Nozzle 1 (ms)	**Nozzle 2 (ms)**	**Nozzle 3 (ms)**	**Nozzle 4 (ms)**
Lime	588	1652	2000	0
Orange	2000	0	0	1071
Lemon	847	2000	486	223
Mandarin	350	0	0	2000
Grapefruit	2000	0	0	1576